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The use of advanced statistical analysis and machine learning methods in Primary Aldosteronism studies

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is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

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List of abbreviations

ACTH	----------	Adrenocorticotropic hormone
AI	----------	Adrenal insufficiency
APA	----------	Aldosterone producing adenoma
ARR	----------	Aldosterone/renin ratio
AVS	----------	Adrenal venous sampling
BAH	----------	Bilateral adrenal hyperplasia
CT	----------	Computed tomography
ES	----------	Endocrine Society
LDA	----------	Linear discriminant analysis
LC-MS/MS	----------	Liquid chromatography / mass spectrometry
MR	----------	Mineralocorticoid receptor
PA	----------	Primary Aldosteronism
PASO	----------	Primary Aldosteronism Surgery Outcome

List of publications

- Wang, X., Heinrich, D.A., Kunz, S.L., Heger, N., Sturm, L., Uhl, O., Beuschlein, F., Reincke, M. and Bidlingmaier, M., 2021. Characteristics of preoperative steroid profiles and glucose metabolism in patients with primary aldosteronism developing adrenal insufficiency after adrenalectomy. Scientific Reports, 11(1), pp.1-10.
- Meyer, L.S., Wang, X., Sušnik, E., Burrello, J., Burrello, A., Castellano, I., Eisenhofer, G., Fallo, F., Kline, G.A., Knösel, T. and Kocjan, T., 2018. Immunohistopathology and steroid profiles associated with biochemical outcomes after adrenalectomy for unilateral primary aldosteronism. Hypertension, 72(3), pp.650-657.
- Heinrich, D.A., Adolf, C., Holler, F., Lechner, B., Schneider, H., Riester, A., Nirschl, N., Sturm, L., Wang, X., Ladurner, R. and Seidensticker, M., 2019. Adrenal insufficiency after unilateral adrenalectomy in primary aldosteronism: long-term outcome and clinical impact. The Journal of Clinical Endocrinology & Metabolism, 104(11), pp.5658-5664.

1. Contribution to the publications

1.1 Contribution to paper I

I am the first author of paper I. My contribution is mainly in:

- Data cleaning
- Conducted all statistical analysis including, but not limited to:
    a. Descriptive analysis
    b. Multiple variates analysis
    c. Predictive modeling and cross-validation by machine learning methods
- Results interpretation
- Manuscript writing and revision

1.2 Contribution to paper II

I am the second author of paper II. My contribution is mainly in:

- Linear Discriminant analysis and results interpretation
- Review manuscript

1.3 Contribution to paper III (Appendix)

I am a co-author of paper III. My contribution is mainly in:

- Statistical analysis
- Review manuscript

2. Introductory summary

2.1 -- Introduction

Hypertension is a major cardiovascular risk factor, which is estimated to affect 70% of the population over 60 years old in the world. Primary aldosteronism (PA) is recognized as the most frequent and curable form of secondary hypertension and is associated with morbidity and mortality. PA used to be considered as a so-called “rare” disease until more and more hypertensive patients were recommended for screening and subsequently diagnosed as being affected with PA. Over the last decades, many studies have been investigating in many respects PA (e.g. prevalence, diagnosis, treatments, outcomes, etc.). One key aspect in diagnosis and treatment is the quality of the analytical methods used to determine hormone concentrations. In recent years, steroid analysis by liquid chromatography/mass spectrometry (LC-MS/MS) became more routinely available at major clinical centers. In contrast to the immunoassays used before, LC-MS/MS allows the measurement of multiple steroids at the same time. Some studies reported potential advantages of using steroid profiles for diagnosis, but few studies exist to evaluate the potential of steroid profiles during initial therapy and long-term follow-up. This dissertation focuses on analyzing the mineralocorticoid and glucocorticoid excess production using LC-MS/MS to improve the decision-making of treatment of unilateral PA to achieve better outcomes and decrease the potential risk of the occurrence of other diseases after treatment.

2.1.1 -- Prevalence of PA

In 1955, primary aldosteronism (PA) was discovered in a 34 year’s old housewife with 4-year hypertension history by Dr. Jerome W. Conn (Conn, 1955). Since then, PA has been considered as a rare disease and underdetected for decades, due to few hypertensive patients being screened. However, the high prevalence has always been detected in organized screening. In 2006, the first large prospective study for the prevalence of PA shows that 11.2% of newly diagnosed hypertensive patients have PA (Rossi et al., 2006). In 2008, the Endocrine Society (ES) guidelines suggested screening PA in the hypertensive patients,

who belong to higher risk groups, and those with hypokalemia (Funder et al., 2008). The ES guideline admitted the high prevalence of PA in 2016 and expanded the higher risk groups, which included hypertensive patients with obstructive sleep apnea (Funder et al., 2016). On the contrary, a retrospective study of general practitioners in Italy and Germany, carried out by web questionnaire, reported that PA is still treated as a rare disease and only 7% to 8% of hypertensive patients are screened for PA, with 1% to 2% prevalence of PA among hypertensive patients (Mulatero et al., 2016). These numbers strongly disagreed with the prevalence from systematically screening hypertensive patients, which is 5.9% (Monticone et al., 2017).

The prevalence of PA differs in various groups. A prevalence of 11.3% was demonstrated in patients with Diabetes. 6% was observed in the general hypertensive population and 11% in referral centers (Buffolo et al., 2017). The prevalence of PA was reported to increase with the severity of hypertension, from 2%-4% in stage I hypertension to 8%-16% in stage II, furthermore 12%-19% in stage III hypertension (Monticone et al., 2012, 2017; Mosso et al., 2003). In patients with resistant hypertension, the prevalence of PA is up to 20% (Calhoun et al., 2002). Patients with hypokalemia, which was the premise of screening for PA, have a 28% prevalence of PA (Gruber and Beuschlein, 2020). The highest prevalence, 34%, was confirmed in the population with hypertension and obstructive sleep apnoea, who are recommended to be screened in 2016 by ES guidelines (Funder et al., 2016).

2.1.2 -- Diagnosis and treatment of PA

According to ES guidelines in 2016 (Funder et al., 2016), the hypertensive patients, who belong to the risk groups, first test the plasma aldosterone/renin ratio (ARR), to detect the possibility of PA. Patients with negative ARR results are unlikely to have PA, therefore they are excluded from here. The rest of the patients receive one or more confirmatory tests to ensure whether the patient has PA or not. No confirmatory tests are needed for patients with spontaneous Hypokalemia, plasma renin below detection level, and aldosterone concentration >20ng/dL. All PA confirmed patients undergo adrenal computed tomography (CT) to exclude adrenocortical carcinoma and assess adrenal venous anatomy. If the surgery is not feasible, then medical treatment with

mineralocorticoid receptor (MR) Antagonist is recommended. If patients can undergo surgery physically and mentally, adrenal venous sampling (AVS) should be done by a proficient radiologist to distinguish between unilateral and bilateral adrenal disease. For bilateral adrenal hyperplasia (BAH), medical treatment with MR antagonist is recommended. However, laparoscopic adrenalectomy is recommended for unilateral adrenal disease, which is caused by aldosterone producing adenoma (APA). Young patients (<35 years) with spontaneous hypokalemia, marked aldosterone excess and CT confirmed unilateral adenoma can undergo laparoscopic adrenalectomy without AVS. In practice, the specific steps can be slightly different (Fig.1) (Holler et al., 2019).

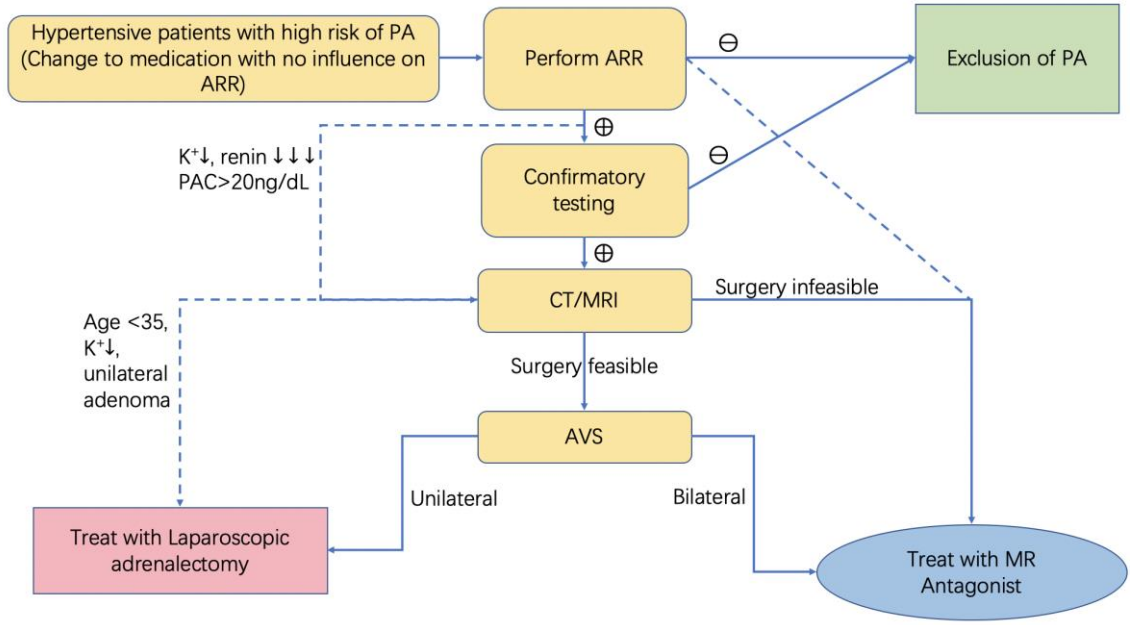

Fig. 1 Algorithm for diagnostic work-up of primary aldosteronism

2.1.3 -- Outcome after treatments

As mentioned above (Fig. 1), if BAH is the cause of aldosterone excess, the recommended treatment is with MR antagonists, while the treatment of choice is unilateral adrenalectomy if the cause is APA (and surgery is feasible). My research focused on APA.

The widely accepted classification of outcomes of APA treatment was defined by the Primary Aldosteronism Surgery Outcome (PASO) study as complete success, partial success, and absent success. Both biochemical and clinical outcomes were taken into account (Williams et al., 2017). If a patient gets normal blood pressure without antihypertensive medication, normal ARR, and normal

potassium values 6-12 months after surgery, then the treatment achieved complete success in this patient. If the blood pressure, hypokalemia is unchanged and ARR decreases smaller than 50%, then the success is absent. The rest are with partial success. The patients with partial success got improved in the correction of blood pressure and hypokalemia but not back to the normal level, and ARR decreased more than 50% (Table 1) (Holler et al., 2019).

	Clinical outcome	Biochemical outcome
Complete success	Normal blood pressure without antihypertensive medication	No hypokalemia, normal ARR
Partial success	Lower blood pressure with the same or less amount of antihypertensive medication or unchanged blood pressure with less antihypertensive medication	No hypokalemia, more than 50% decline in raised ARR
Absent success	Unchanged or increased blood pressure with the same or more antihypertensive medication	Persistent hypokalemia, persistently raised ARR

Table 1. The definition of complete, partial, and absent success in clinical and biochemical outcomes (Holler et al., 2019)

Despite the rate of biochemical complete success is very high, 94%-100%, the total heal of hypertension, which is defined as complete clinical success, is only around 50% or even less (Muth et al., 2015; Steichen et al., 2012; Volpe et al., 2015; Williams et al., 2017). The problem of improving the rate of clinical success is still open for solutions.

In recent years it was discovered that many PA patients have both mineralocorticoid and glucocorticoid excess (Arlt et al., 2017). Unilateral adrenalectomy decreases the secretion of both mineralocorticoid and glucocorticoid. Early studies reported the incidence of hypotension in about a third of the patients two years after unilateral adrenalectomy, which was suspected to be caused by the reduced cortisol secretory capacity of the

remaining adrenal gland (Gordon et al., 1989a, 1989b). The adrenocorticotropic hormone (ACTH) stimulated cortisol concentrations after surgery have been demonstrated to be lower compared to the situation before surgery (Honda et al., 2013). 29% of patients were observed with adrenal insufficiency, which is defined as low cortisol secretory responding to ACTH stimulation (Arlt et al., 2017). Adrenal insufficiency without correction affects the well-being and life quality of patients, even endangering life in extreme cases. The occurrence of adrenal insufficiency after surgery should be considered when decisions are made between surgery or medical treatment for patients with APA. Medical treatment should be provided to patients with a high risk of adrenal insufficiency after surgery. Yet, adrenal insufficiency after unilateral adrenalectomy in patients with PA is still not emphasized worldwide. More studies in this field are required, to identify or even predict adrenal insufficiency in time.

2.2 -- Bottleneck of PA research

The difficulty of detecting PA, caused by the low examination rate in hypertensive patients, leads to the rareness of PA patients, increasing obstacles in PA research. The limited size of data is a common issue for rare diseases. For decades, PA researchers were confined to analysis of basic information, physical examination, clinical chemistry, and hormone assessments in a low number of patients (Schirpenbach and Reincke, 2006). As technology rapidly develops, LC-MS/MS is widely used in chemical analysis, due to its high specificity and sensitivity (Vogeser and Parhofer, 2007).

2.2.1 -- Steroid profiling by Liquid chromatography/mass spectrometry

Steroid profiling is a steroid panel, consisting of 15 steroids, measured by LC-MS/MS. It not only provides the concentration of multiple steroids simultaneously with high sensitivity and specificity but also largely increases the information that can be acquired from a limited volume of samples (Shackleton, 2010). Steroid profiling brings a new way of investigating the pathophysiological mechanism of PA (Eisenhofer et al., 2016; Holler et al., 2019; Peitzsch et al., 2015). It displays more information on the connection to multiple-steroid patterns instead of a single steroid.

Hypothesis tests and descriptive statistical analysis have been the common way of analyzing and presenting data in medical research (Cohen et al., 2021; Monticone et al., 2018; Schweitzer et al., 2019; Taylor et al., 2017). High dimensional data, measured by LC-MS/MS, brings new challenges to the interpretation and visualization of data. The descriptive statistical analysis and hypothesis testing are not competent to analyze multiple-steroid patterns.

2.2.2 -- Advanced statistical analysis

LC-MS/MS produces plenty of data. We can't interpret it the old way. In recent years, the state of art statistics are used to interpret data in adrenal disease (Küpers et al., 2012; Schweitzer et al., 2019; Turcu et al., 2020). Here, I briefly introduce three commonly used analyses:

1. Logistics regression is widely used in two groups classification when data is linearly separable. The basic form of logistic regression uses a logistic function to find a relationship between one or more independent variables and the probability of the particular outcome. Complex extensions for separating three or more groups exist (Hosmer et al., 2013).

2. Linear discriminant analysis (LDA) is an approach to classify the observations into two or more groups by determining a linear combination of features. LDA is commonly used to reduce dimensions. The linear combination may be suggested to be the linear classifier (Balakrishnama and Ganapathiraju, 1998).

3. A decision tree is a chart of decision procedures. Fig. 2 is a simple example with one internal node. An internal node is a test with the answer "Yes" or "No". "Yes" and "No" are two branches, which lead to different leaf nodes, namely decisions. Compared to other methods, the decision tree is simple to understand and uses a white-box model.

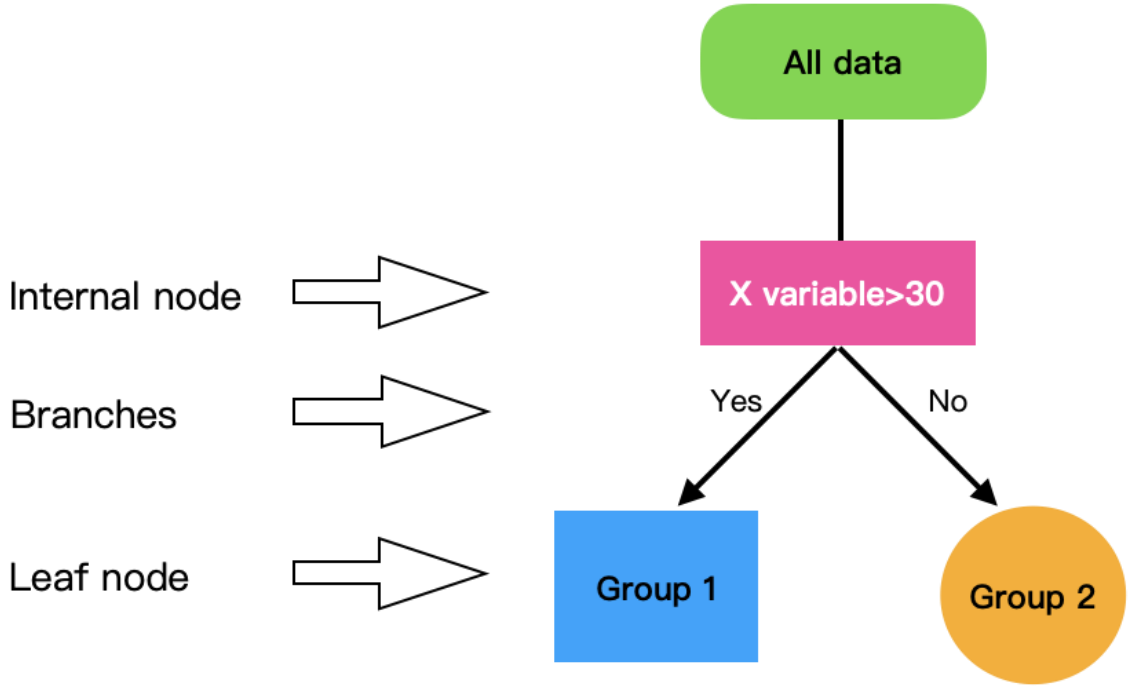

Fig. 2 An example of a decision tree.

Besides these three, more sophisticated approaches are used in paper II to interpret our data, such as random forest, model-based boosting, and so on.

Validation is a crucial part of the analysis, especially when data size is small and complex machine learning methods are used. Because the relationship between the feature and the target variable, which was caught by the model, inside training data may not represent the relationship in the whole population. The model may look very promising in our data, but it can't predict anything in the new data. To prevent this situation, we use cross-validation in the paper I. We separate the data into training sets and validation sets. We train our model in the training set and validate the model in the validation set. In the end, we choose the best model based on the performance in the validation set, not in the training set.

2.3 -- Coverage of this dissertation

Papers in this dissertation cover 3 main aspects of PA research in this dissertation, which are subtyping of PA, outcomes of PA, and the application of steroid profiling in PA research (Table 2). Three papers are all concentrated on understanding and improving the outcomes of unilateral adrenalectomy in different aspects. Paper I and II utilized steroid profiling and advanced statistical analysis to interpret the outcomes of APA treatment and demonstrate the latent

power of steroid profiling on the research of PA. In addition, paper II gives another possible criterion of subtyping PA and the potential tool for it.

	Subtyping	Outcomes	Steroid profiling
Paper I		Yes	Yes
Paper II	Yes	Yes	Yes
Paper III		Yes	

Table. 2 Perspectives covered by 3 papers.

2.3.1 -- Brief intro of papers

Each paper in this dissertation answered some questions in PA research. Some answers pointed out new possible research directions. Some answers confirmed the contribution of new technology in conquering difficulties in PA research. Some answers brought light to the status that has been omitted by many PA researchers. The following questions and context of each paper provide a better understanding of the full picture of our research and connections between the papers. My main contribution was to paper I. However, chronologically and logically, the order should be paper II, paper III, and paper I.

Paper II. Immunohistopathology and steroid profiles associated with biochemical outcomes after adrenalectomy for unilateral primary aldosteronism

- Is there any difference among the complete, partial, and absent success groups for histopathology or steroid concentrations?
- Can steroid profiling before surgery correctly classify patients according to their outcome after surgery?
- Are specific patterns of steroid profiles associated with distinct histopathological phenotypes?

Paper II focused on using the new technology, steroid profiling, to improve the prediction of outcomes after surgery. The investigation of immunohistopathology and steroid profiles of these patients help understand the pathology of PA and have the potential to improve the diagnosis of subtypes. Therefore, a higher rate of complete success after surgery can be achieved.

Paper III. Adrenal Insufficiency After Unilateral Adrenalectomy in Primary Aldosteronism: Long-Term Outcome and Clinical Impact

- What is the prevalence of postoperative adrenal insufficiency?
- How severe is postoperative adrenal insufficiency?
- Do patients with adrenal insufficiency after surgery need treatment and how long?

Paper III draws attention to cortisol production after surgery. The adrenal insufficiency after unilateral adrenalectomy in patients with PA is normally assumed as a transit phenomenon. This paper investigated the cortisol level in the long term, to gain more understanding of the AI and provide prompt treatment.

Paper I. Characteristics of preoperative steroid profiles and glucose metabolism in patients with primary aldosteronism developing adrenal insufficiency after adrenalectomy

- Any association between steroids before surgery and post-operative adrenal insufficiency (AI) can be identified?
- Can adrenal insufficiency be predicted before surgery?
- Can preoperative steroid profiling improve the prediction of postoperative AI?

Paper I investigated the association between preoperative steroid profiles and the occurrence of AI after unilateral adrenalectomy in patients with PA. It has been reported that about a third of patients after unilateral adrenalectomy displays low ACTH stimulated cortisol level, AI. LC-MS/MS measured steroid profiles have been demonstrated with a positive influence on the diagnosis of PA. This paper looked into the preoperative steroid profiles by advanced statistical analysis to detect any hints for postoperative ACTH stimulated cortisol concentrations.

2.4 -- Summary

This dissertation illustrated two different points of view on outcomes of unilateral adrenalectomy, which is the treatment of unilateral PA. Firstly, according to the PASO studies (Williams et al., 2017), the outcomes are defined as complete, partial, or absent success according to the extent of normalization of blood pressure, the remission of raised ARR, and the correction of hypokalemia.

Secondly, the outcomes are defined from the perspective of cortisol level after surgery. It has been reported by other papers (Arlt et al., 2017) and confirmed by our cohort, that around 30% of patients suffer from adrenal insufficiency after surgery. Once they are neglected, it leads to hypotension or adrenal crisis (Gordon et al., 1989a, 1989b).

Moreover, this dissertation demonstrated the utility of steroid profiles, which are measured by LC-MS/MS, in predicting the outcomes of surgery in both outcome systems. The complete, partial, and absent success groups are correctly classified by the linear discriminant analysis and random forest models with 76%-86% accuracy. Models with steroid profiles performed better than models without steroid profiles in the prediction of adrenal insufficiency.

Last but not the least, this dissertation identified a potential predictor for predicting adrenal insufficiency, which is insulin at 60 minutes during OGTT. This can be related to glucocorticoid co-secretion, which has been reported in 2017 (Arlt et al., 2017). More studies are ongoing in understanding the latent association between insulin and PA (Adler et al., 2020).

More studies have indicated the contribution of steroid profiling in the prediction of outcomes in PASO standards after paper II was published (Burrello et al., 2020). At the same time, steroid profiles showed their power in predicting genotypes of APA as well (Williams et al., 2016). Knowing the mutations of the tumor helps understand the pathology of PA and guides the treatment. In some cases, steroid profiling has been used in the diagnosis of PA and led to complete success in clinical and biochemical outcomes (Holler et al., 2019). We can expect soon steroid profiling will be added to the workflow for diagnosis and decision-making of treatment of PA (Fig 3.).

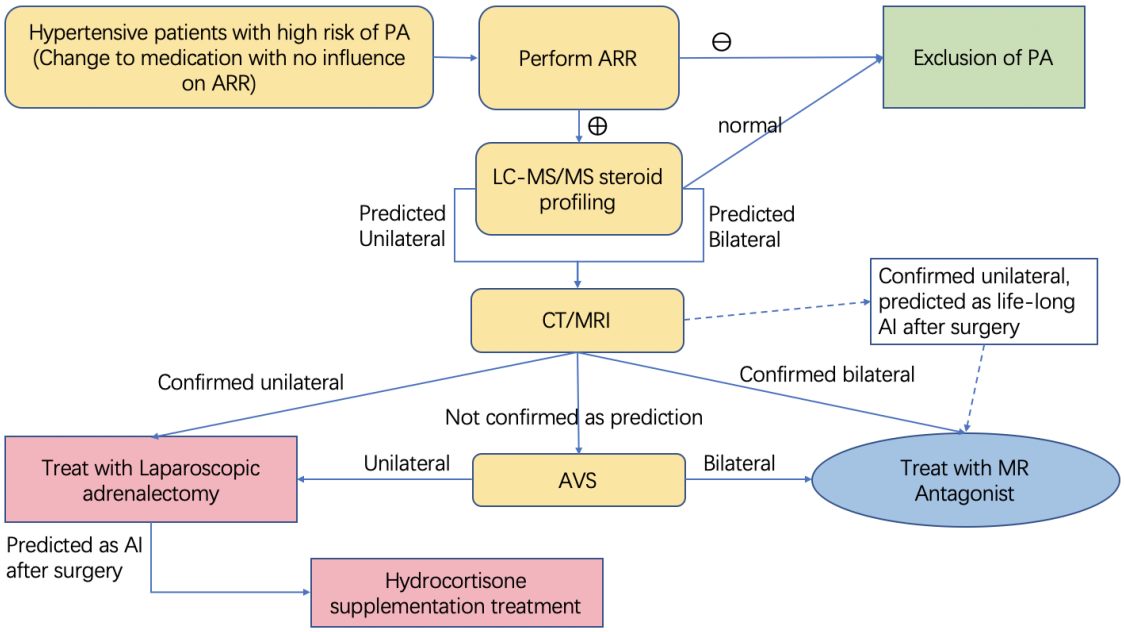

Fig. 3 Future algorithm of diagnosis of primary aldosteronism. The dashed line represents it depending on the individual situation.

Paper I

Characteristics of preoperative steroid profiles and glucose metabolism in patients with primary aldosteronism developing adrenal insufficiency after adrenalectomy

Author: Xiao Wang, Daniel A. Heinrich, Sonja L. Kunz, Nina Heger, Lisa Sturm, Olaf Uhl, Felix Beuschlein, Martin Reincke & Martin Bidlingmaier

Sci Rep 11, 11181 (2021). https://doi.org/10.1038/s41598-021-90901-4

Paper II

Immunohistopathology and steroid profiles associated with biochemical outcomes after adrenalectomy for unilateral primary aldosteronism

Author: Meyer LS, Wang X, Sušnik E, Burrello J, Burrello A, Castellano I, Eisenhofer G, Fallo F, Kline GA, Knösel T, Kocjan T, Lenders JWM, Mulatero P, Naruse M, Nishikawa T, Peitzsch M, Rump LC, Beuschlein F, Hahner S, Gomez-Sanchez CE, Reincke M, Williams TA.

Hypertension. 2018 Sep;72(3):650-657.

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Apendix A:

Paper III

Adrenal Insufficiency After Unilateral Adrenalectomy in Primary Aldosteronism: Long-Term Outcome and Clinical Impact

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